The University of Chicago

THE ALTMANN TECHNIQUE FOR FIXATION BY DRYING WHILE FREEZING

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY

By

ISIDORE GERSH

Private Edition, Distributed by
THE UNIVERSITY OF CHICAGO LIBRARIES
CHICAGO, ILLINOIS

Reprinted from
THE ANATOMICAL RECORD
Vol. 53, No. 3, 1932

The University of Chicago

THE ALTMANN TECHNIQUE FOR FIXATION BY DRYING WHILE FREEZING

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANATOMY

By

ISIDORE GERSH

Private Edition, Distributed by
THE UNIVERSITY OF CHICAGO LIBRARIES
CHICAGO, ILLINOIS

Reprinted from
THE ANATOMICAL RECORD
Vol. 53, No. 3, 1932

R. UNIV.
BIBLIOTHEEK
LEIDEN

Reprinted from THE ANATOMICAL RECORD
Vol. 53, No. 3, August, 1932

THE ALTMANN TECHNIQUE FOR FIXATION BY DRYING WHILE FREEZING

I. GERSH[1]
Hull Laboratories, Department of Anatomy, The University of Chicago

TWO FIGURES

CONTENTS

Method 310
A. General considerations 310
B. Apparatus 312
C. Explanation of the apparatus 312
D. Technique 319
E. Safety precautions 323
Results 324
A. Fixation 324
1. General considerations 324
2. Peculiarities of fixation by the Altmann technique 327
3. Examples of fixation by the Altmann technique 329
B. Histochemisry 331
1. General considerations 331
2. Application of the Altmann technique to histochemistry 334
C. Permanent preparation of supravitally stained organs 335
Conclusions 336
Literature cited 337

---

[1] We wish to express our gratitude to Dr. Robert R. Bensley for suggesting the Altmann method as a means of overcoming certain difficulties in histochemistry, for his innumerable valuable suggestions in the course of the work, but chiefly for the kind and generous encouragement he offered throughout the investigation. We are greatly indebted to Dr. Edward J. Stieglitz for his assistance, interest, and suggestions. We are also indebted to Dr. Warren C. Johnson, of the Department of Chemistry, University of Chicago, for his invaluable suggestions and cooperation—the apparatus would not be developed to its present stage without his advice. We wish to acknowledge gratefully the

INTRODUCTION

Over forty years ago, Altmann introduced an ingenious method for the histologic fixation of tissues. He froze organs or blocks of tissues and dehydrated them at low temperatures (— 15°C. to — 20°C.) in a vacuum. He reported that the method resulted in the fixation of tissues without shrinkage, that the method would be suitable for the accurate localization of substances normal to the organism or introduced into the body, and, that by means of the technique, accurate localization of intravital dyes with the production of permanent preparations would be possible. The method has received scant notice from histologists, and has been simply referred to by Mann, Mathews, Bayliss, and Romeis. Undoubtedly, this failure to recognize and develop such an important tool was due to the difficulties involved in making a piece of apparatus that would not be too time-consuming or require too much effort.

We have attempted to overcome these difficulties. The apparatus, though expensive, requires very little attention and can be used to dehydrate a large number of blocks at one time. We have attempted to probe the possibilities of the method, and have confirmed the predictions of Altmann. The method and some of the results will be described in this paper. Subsequent papers will show the efficiency of the method when applied to specific problems involving fixation and microchemistry, as well as intravital and supravital staining.

METHOD

A. General considerations

The principles on which the Altmann technique is based are very simple. When blocks of tissue are maintained at a

assistance of Dr. Normand L. Hoerr and Mr. Edgar I. Eisenstaedt, who made conditions bearable in the earlier part of the investigation, of Miss Agnes Nixon for the diagram of the apparatus, and Mr. Edgar I. Eisenstaedt again for the photograph of the apparatus.

Acknowledgment is made to the Chemical Foundation for its Fellowship and support received from its Julius Stieglitz Fund for Research in Chemistry Applied to Medicine, which made this work possible.

temperature of —20°C., the temperature is far lower than the freezing point of water containing salts in solution in the concentration occurring in the body fluids and tissues. The result is that, the water being frozen, there is no fluid medium in the block which will allow any appreciable diffusion of substances within the block. Water at —20°C. has an appreciable vapor pressure (0.9441 mm. mercury). Therefore, if the block of tissue is placed in a vacuum chamber which is maintained at —20°C., and a vacuum of 1×10^{-4} mm. is established, the vapor pressure of the ice in the block is considerably higher than the vapor pressure of water in this atmosphere. The water will thus gradually go over into the vapor state and leave the block dehydrated. Partly to speed up the production of the vacuum and to maintain it, but chiefly to avoid contaminating the oil of the vacuum pump, a dehydrating agent (phosphorus pentoxide) is inserted between the dehydrating chamber and the pump.

To maintain the temperature of the vacuum dehydrating chamber at —20°C., we have had to resort to two methods. One method is to surround the dehydrating chamber with a Dewar flask containing acetone and carbon dioxide snow ('dry ice'). The fact that the snow must be added at very short intervals (every fifteen to thirty minutes) for two days to maintain the temperature within the narrow limits of —20° to —25°C. makes this method of chilling laborious and impractical.

The other method of maintaining the temperature is by far the more desirable. The boiling point of liquid ammonia at atmospheric pressure (760 mm.) is —33.7°C. By increasing the pressure on the liquid, the boiling point is raised. So that at a pressure of 1304.6 mm. (760 mm. plus 544.6 mm. at sea level) the boiling point is —22°C. The liquid ammonia is poured into a Dewar flask, hermetically sealed to the dehydrating chamber (vacuum tube) which is immersed in the liquid ammonia and is permitted to boil off under a pressure of 54 cm. of mercury through a tube connected with the usual mercury manometer arrangement. The ammonia vapor is

passed out of the building through a rubber tube attached to the mercury pressure tube.

Though the temperature of the ammonia is at —22°C., the temperature of the inside of the dehydrating chamber was found empirically to be close to —20°C., and to vary from it only by one degree in either direction.

The density of water at body temperature (37° to 39°C.) is approximately the same as that of ice at about —20°C. Therefore, when a block of tissue is maintained at about —20°C., and fixed by means of the Altmann apparatus by the removal of water, one would expect the volume of the block of tissue to be unchanged after fixation. In other words, theoretically, there should be no shrinkage at all during the process of fixation itself.

The last part of the procedure consists of the infiltration of the block of tissue with melted paraffin, or celloidin.

To facilitate the explanation of the apparatus as we have developed it up to the present, a detailed diagram will be given. This will be followed by a description of the different procedures used and the specific purpose of each step, together with an account of some of the results obtained by means of the Altmann technique.

B. Apparatus

The apparatus we have used for the Altmann technique is described in figures 1 and 2. To facilitate the building of a similar piece of apparatus, to give the reason for the particular form or size of some of the parts of the apparatus, and to assist the operator in his manipulations, we will discuss some phases of the apparatus in the following section.

C. Explanation of the apparatus

The mercury-vapor pump. The mercury-vapor pump is a one-stage simple mercury-vapor pump heated by a Bunsen burner clamped into position, whose flame is protected by a chimney fitting closely under the bulb of the vapor pump.

We think that the slightly lower pressure that could be obtained with a three-stage mercury-vapor pump in contrast to the single-stage pump would not shorten the time required for dehydration enough to justify the added expense. (For the theory and practice of the mercury-vapor pump, the oil pump, the MacCleod gauge, etc., see references on vacuum technique, especially Dunoyer, Dushman, Hoag, and Newman.)

The oil pump. The oil pump which backs up the mercury-vapor pump is a Cenco Hyvac Pump run by a $\frac{1}{6}$-H.P. electric motor. Both pumps are kept continuously functioning during the period of dehydration.

The stopcock (*A*). The stopcock between the mercury-vapor pump and the phosphorus-pentoxide tube is for use only in case of emergency or when the vacuum pump is not in use. The bore of the stopcock should be approximately the same as that of the tube on either side.

The phosphorus-pentoxide chamber (*C*). Phosphorus pentoxide is used as a drying agent to pick up the water as it comes over in the vapor state and thus to prevent contamination of the oil in the vacuum pump with condensed water. Enough phosphorus pentoxide is placed in the chamber to cover the length of the tube to half its height when in the dry state. For convenience in reloading, a wide-mouthed glass stopper is placed at one end of the tube. When the phosphorus pentoxide is hydrated to the extent that most of the material is a thick gummy liquid, the stopcock (*A*) between the main line and the dehydrating chamber is turned off, the vacuum in the line is broken, the gummy liquid is scraped out and is replaced with anhydrous phosphorus pentoxide. The vacuum is reestablished and the stopcock is turned on again. Generally, the amount of phosphorus pentoxide that is added will take up about 10 to 15 grams of water.

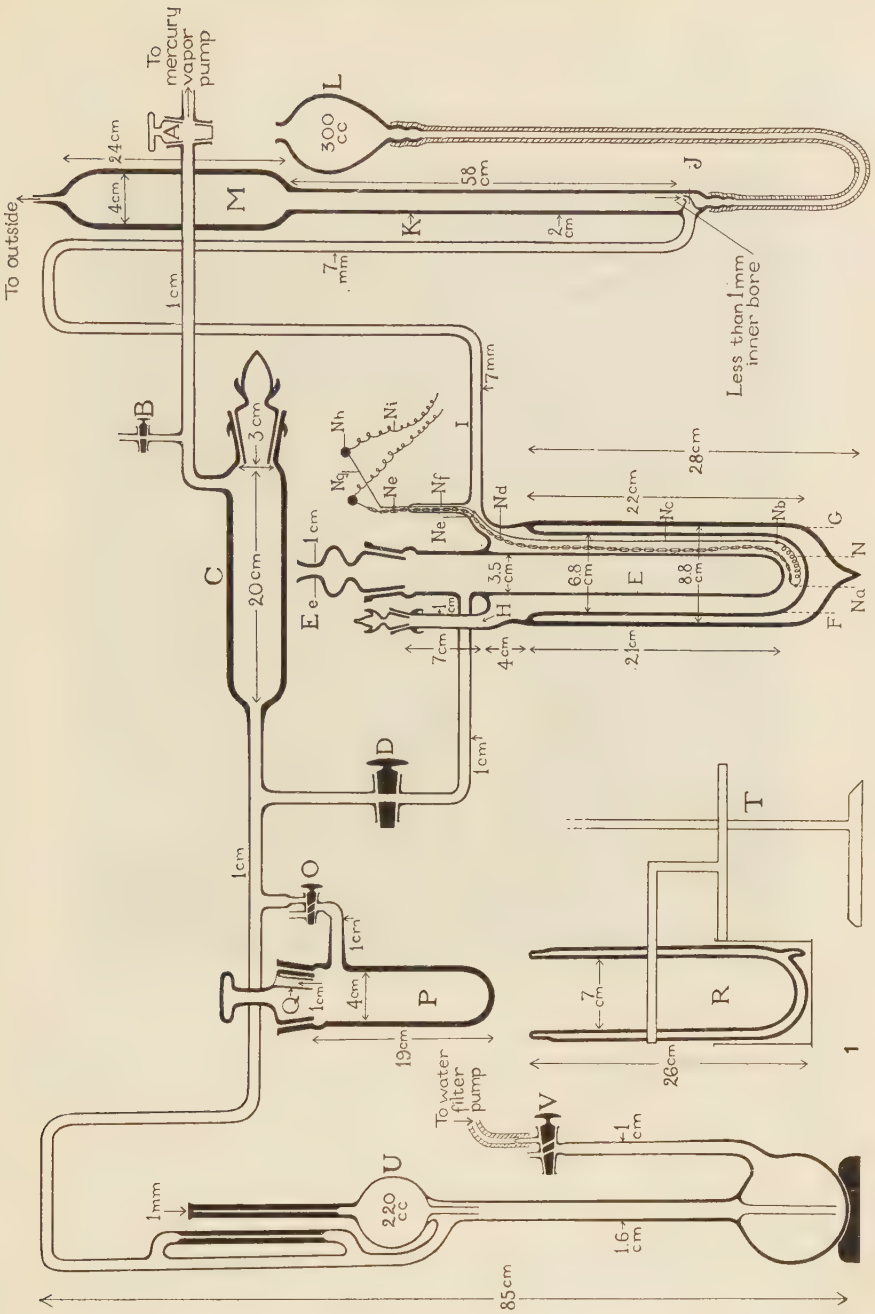
The dehydrating chamber and the surrounding Dewar flask. The dimensions of the apparatus are such that it is necessary to add liquid ammonia to the Dewar flask only once every

twelve hours. The level of the liquid ammonia should be above the uppermost of the three or four trays in the dehydrating chamber. The blocks of the organs are placed in these trays, which are made of copper mesh. They are 1.5 to 2.0 cm. high, and are of such a diameter as to just fit the inside of the dehydrating chamber. These are lowered to, and raised from, the bottom of the dehydrating chamber by means of a copper wire soldered to the outer edge of the

Fig. 1 Diagram of the glass system used in the Altmann technique.

A is the stopcock interposed between the mercury vapor pump (not shown in this figure) and the main line of the vacuum system. The stopcock, *B*, is used to break the vacuum and opens to the outside air. Water vapor entering the system when this is open to the air is taken up by the phosphorus pentoxide in the chamber, *C*. The stop cock, *D*, is interposed in the main line, and during embedding separates the dehydration chamber, *E*, from the main line. The ground-glass stopper of the dehydration chamber has an opening in it, *Ee*, into which a thermometer may be sealed with de Kotinsky wax. The dehydration chamber, *E*, is surrounded by the Dewar flask whose inner wall, *F*, and outer wall, *G*, are shown in the figure. Liquid ammonia is poured into the Dewar flask, *FG*, through the inlet tube, *H*, which is closed by a ground-glass stopper held in place with two rubber bands. The outlet tube, *I*, serves for the escape of ammonia gas, and ends in the capillary tube, *J*, which in turn opens into the mercury-pressure tube, *K*. This tube, *K*, is connected with rubber tubing to the mercury manometer bulb, *L*. The ammonia gas bubbles passing through the mercury in the mercury-pressure tube, *K*, leave through the enlarged safety tube, *M*, and are led off to the ouside by rubber hose connected to the safety tube, *M*. The heating unit in the liquid ammonia Dewar, *FG*, consists of a 'chromel-alumel wire' coil, *N*, which is connected by soldered joints, *Na*, *Nb*, to the nickel wires, *Nc*. The nickel wires, *Nc*, are insulated from each other by glass beads, *Nd*, surrounding one of them. The nickel wires, *Nc*, are joined with silver solder, *Ne*, to tungsten wires, *Nf*, which pass through the glass tubing and are joined to nickel wire again, *Ng*, by silver solder, *Ne*. The nickel wires, *Ng*, are attached to binding posts, *Nh*, which are also connected with copper wires, *Ni*, leading to a rheostat and 6-volt transformer, not shown in the picture. The transformer is connected by a switch to a 110-volt A-C circuit.

Separated also from the main line by a two-way stopcock, *O*, is the embedding chamber, *P*, whose ground-glass stopper contains a tube, *Q*, sealed to it to admit a thermometer. The embedding chamber, *P*, may be surrounded by a Dewar, *R*, which may be lifted into place by raising the cup in which it rests by some such apparatus as is shown directly to the right, at *T*. The vacuum used in the operation of the MacCleod gauge, *U*, is controlled by the two-way stopcock, *V*. All of the glass used in the system is of Pyrex. All of the dimensions of the tubing given in the diagram are of the inner-bore diameter or length and do not take account of the thickness of the glass; this is indicated in the diagram by the relative thickness of the lines.


To outside
To mercury vapor pump
24cm
4cm
A
M
L
300 cc
58 cm
J
K
2 cm
7 mm
1 cm
Less than 1mm inner bore
B
3cm
C
20cm
1cm
7mm
I
Nh
Ni
Ng
Ne
Nf
Nd
28cm
22cm
Nc
Nb
G
N
Na
F
E
6.8 cm
8.8 cm
3.5 cm
H
1 cm
Ee
7cm
4cm
21cm
D
1cm
T
O
Q
1cm
1cm
4cm
P
19cm
7 cm
R
26cm
To water filter pump
V
1 cm
U
220 cc
1mm
16 cm
85cm

wire baskets. It was found that the temperature of the material in the tray remains nearly constant at — 20°C. when the liquid ammonia is at or above the level of the highest

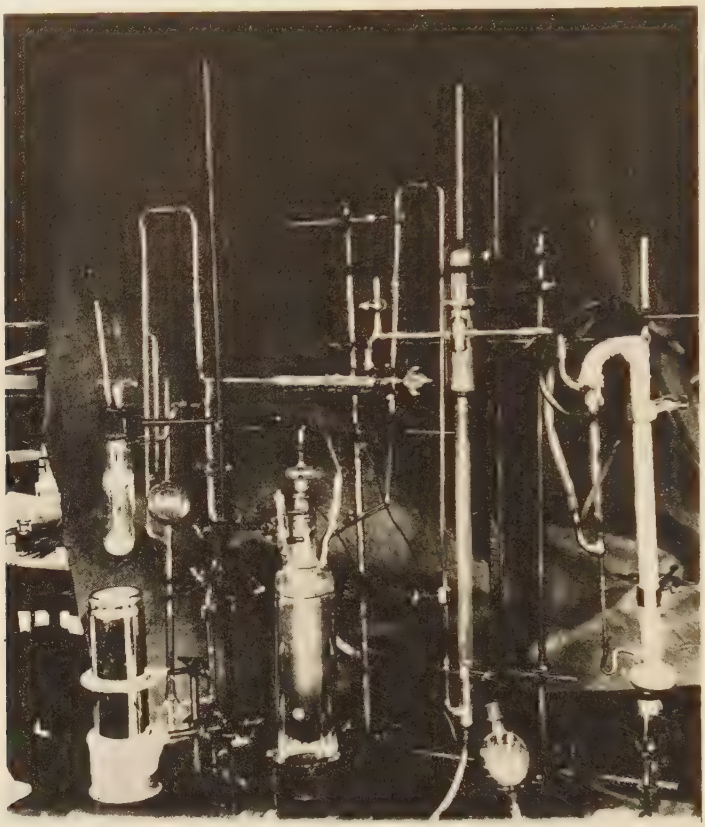


Fig. 2 Photograph of the apparatus to show the actual distribution of the parts.

The insulation around the Dewar flask surrounding the dehydrating chamber has been removed. (The Dewar flask had not yet been silvered.) The mercury-vapor pump, not drawn in figure 1, is shown at the right in this figure.

tray, but not when it is more than 1 cm. below the level of the highest tray.

It is convenient that the cap of the ground-glass stopper of the dehydrating chamber (*Ee*) should have an opening in

it about 1 cm. inside bore and about 3 cm. long for the insertion of a thermometer or other experimental instrument.

The ammonia freezing system. The outlet tube should be no larger than 0.7 cm. inner bore tubing and its highest point should be at least 60 cm. above the capillary opening into the mercury manometer tube. The capillary tube is innersealed into the mercury tube and opens at about the center of it. The capillary opening (*J*) is 1 mm. or less in diameter and is directed upward. The mercury tube itself (*K*) should be of tubing 2 to 2.5 cm. in diameter and 60 cm. in height. It is surmounted by a tube of larger diameter which serves as a safety chamber. The upper end of this widened portion is continued as a narrow tube which is connected to rubber tubing leading the ammonia gas to the outside of the building. The capillary opening into the mercury column assures the breaking up of the ammonia gas into a continuous stream of small gas bubbles; the large bore of this column prevents the mercury from being lifted too high and disturbing the apparatus by the resulting tumult.

To facilitate the transfer of the liquid ammonia, the transfer tube (*H*) should not be less than 1.5 cm. inner bore at the narrowest point.

It is advisable to evacuate the Dewar flask (*FG*) without silvering the inner walls until the operator has become adept at handling the apparatus. When the Dewar is silvered, it should be done in such a way that two streaks opposite each other are unsilvered, to permit of inspection of the relation of the level of the liquid ammonia in the Dewar to the top of the uppermost basket in the dehydrating chamber. The liquid ammonia will last at least three or four times longer in the silvered than in the unsilvered Dewar flask. While the Dewar is not silvered, insulation of the Dewar with a thick pad of cotton is advised. It reduces radiation to such an extent that the liquid ammonia lasts twice as long as without insulation.

When the mercury pressure on the liquid ammonia system is raised, it sometimes requires considerable time for the

ammonia gas to bubble through the mercury column in a steady stream. In other words, the temperature of the liquid ammonia sometimes rises rather slowly to —20°C. This occurs especially when the liquid ammonia has been supercooled and when the Dewar used has been silvered. To speed up the boiling, we have installed a heating unit that is glass-sealed. The current through the coil is broken only after a regularly interrupted stream of ammonia bubbles passes through the mercury in the outlet tube. The heating unit is diagrammed in figure 1.

The embedding chamber (*P*). The embedding chamber is separated from the main line by a two-way stopcock, so that the infiltration of the block with paraffin or celloidin may take place at the same time that the dehydrating chamber is functioning without interfering with the dehydration. The opening in the cap of the embedding chamber should be no less than 1 cm. inside diameter, so that it may accommodate a thermometer or other tube. (The seal of the thermometer to the cap of the embedding chamber and the dehydrating chamber is carefully made of de Kotinsky sealing wax.) It it surrounded by a Dewar flask which can be lowered and removed for the addition of warm water to control the temperature of the embedding chamber.

The MacCleod gauge (*U*). To be certain that there is no leakage in the line, it is necessarry to have some method of determining how low the pressure in the line actually is. The MacCleod gauge used for this purpose has a volume bulb of 220 cc. as a base for the calibrated tube of approximately 1 mm. inner bore. The gauge serves also to ascertain when the blocks are dehydrated. When the pressure in the line is about 1×10^{-4} mm. and remains at this point for at least twelve hours, the blocks of tissue were always found to be dehydrated.

There are several small pieces of accessory apparatus which are not part of either the vacuum system or the freezing system. These accessory tools are described as they are encountered in the course of the description of the technique.

D. Technique

The essential advantage of the Altmann method of fixation over all other known methods is the rapidity of the fixation of the tissues whether for cytological, histochemical, or supra-vital work. Therefore, the necessity for rapidity of movement of the operator cannot be stressed too strongly.

The animal is killed by a single violent blow on the head and immediately dissected for the organ desired. Agonal movements are prevented from interfering with the operator by an assistant who holds the animal tightly stretched. The organ to be studied is removed and sliced with a sharp razor blade. An assistant, who has previously immersed a copper plate in liquid air until boiling has stopped, touches the plate to the slice of tissue, and transfers it directly to the liquid air. The liquid air is contained in a Pyrex silvered, wide-mouthed Dewar flask. The block remains immersed in liquid air until it separates of itself from the copper plate, which it does when it is cooled to the temperature of the liquid air.

The blocks of tissue usually float on the surface of the liquid air. If they fail to come to the surface of the liquid air, it is exceedingly difficult to raise them when they are to be transferred to the dehydrating chamber. To avoid this difficulty, a copper screen is suspended by wires in the liquid air about two inches below the surface in such a way that when the blocks sink into the liquid air, raising the copper screen by means of the wires lifts the blocks of tissue with the screen. Manipulations are speeded up and confusion avoided by this simple device.

The copper plate used in handling the tissue is 5 mm. wide and 2 cm. long and is soldered to a copper wire the other end of which is bent so as to hang from a rod clamped over the Dewar flask containing the liquid air. Three or four of these plates can be used to freeze successively twenty or more blocks of tissue in rapid order.

As soon after freezing in liquid air as possible, the blocks of tissue are transferred to the copper trays described above. These trays are then quickly transferred to the dehydrating

chamber, which has been cooled to the temperature of liquid ammonia by the addition of liquid ammonia to the surrounding Dewar flask. The liquid ammonia is added to the Dewar flask (*FG*), surrounding the dehydrating chamber as follows:

Liquid ammonia is transferred from a liquid-ammonia tank to a Pyrex Dewar flask, which may or may not be silvered. This Dewar flask is covered with a close-fitting rubber stopper. Passing through this rubber stopper are two glass tubes, one passing to the bottom of the Pyrex Dewar flask, the other barely beyond the lower part of the stopper. A rubber bulb is used to force air through the smaller tube which causes the liquid ammonia to pour out of the larger tube through the inlet tube (*H*) into the Dewar flask (*FG*).

During the transfer of the copper trays to the cooled dehydrating chamber, the temperature of the blocks in the trays never rose above —50°C. in the series tested. After all of the trays are transferred to the dehydrating chamber, and the stopcocks, etc., are so disposed as to make a closed system, the oil pump is started, and soon afterward the mercury-vapor pump.

The column of mercury in the mercury pressure tube (*K*) is then raised rapidly to 54 cm. by raising the mercury bulb (*L*), and the current is turned on connecting the heating coil in the liquid ammonia. As soon as a steady stream of ammonia bubbles is passing through the mercury column, the current is switched off. The increase in pressure raises the temperature of the dehydrating chamber to about —22°C. The apparatus is now permitted to run without further change of the vacuum system until the blocks of tissue are considered to be dried.

From time to time, readings are made of the pressure of the vacuum system in millimeters by means of the MacCleod gauge. After the pressure has remained at 1×10^{-4} mm. for twelve hours, the blocks may be considered dehydrated.

When the level of the liquid ammonia in the Dewar flask falls to about 1 cm. above the level of the highest tray in the dehydrating chamber, more liquid ammonia should be

added. To add liquid ammonia, the level of the mercury in the mercury manometer tube is first lowered as rapidly as possible to below the level of the capillary tube opening into it. The liquid ammonia in the Dewar flask then boils violently, but generally comes to equilibrium after a lapse of about ten minutes. Nevertheless, the glass stopper of the inlet tube should be removed very carefully.

After the addition of liquid ammonia, which is done in the same manner as described above, the stopper is replaced and fastened to the inlet tube with two rubber bands. Then the pressure on the liquid ammonia is increased by returning the mercury to its former level in order to raise the temperature to about — 20°C. During the whole procedure, the vacuum is, of course, not affected.

After the blocks of tissue are considered to be dehydrated, the vacuum is broken, and the baskets are removed to the air. The blocks of tissue are now ready to be embedded. They may be embedded in paraffin or celloidin. The choice of embedding medium depends on the material investigated. For cytological purposes, paraffin embedding after paraffin oil infiltration in vacuo, or celloidin embedding is best. The preliminary infiltration with paraffin oil may also be used in microchemical work where the substance to be tested for (such as glycogen) is extremely insoluble in paraffin or paraffin oil. In the permanent preparation of supravitally stained tissues where the dye is difficulty soluble, this method is also advisable. Direct paraffin infiltration is necessary for histochemical work with substances that are very diffusible, or with supravital dyes that are soluble.

Direct infiltration in paraffin is the simplest procedure. The embedding chamber is surrounded by a Dewar flask containing water at about 45°C. Melted paraffin is poured in a test tube previously warmed to the melting point of the paraffin, and the dehydrated blocks of tissue are dropped into it. The tube is placed quickly into the embedding chamber, and a vacuum established. After five minutes, the blocks generally sink, the infiltration is usually complete, and the vacuum

may be broken. From this point embedding proceeds as usual.

The more delicate method of infiltrating in paraffin oil is very nearly the same as the direct paraffin infiltration, but is considerably slower. Heavy Stanolax oil is poured in a wide test tube. The tissues are dropped into it, and the test tube with its contents is lowered into the embedding chamber maintained at room temperature. The vacuum is established and maintained for five to ten minutes, depending on the thickness of the desiccated blocks of tissue. After the vacuum is broken, the tissues are removed and placed into melted paraffin. Infiltration with paraffin oil may be accomplished without a vacuum in the course of several hours by immersion of the dried block in the oil with occasional agitation. Several changes of paraffin in the course of about twenty hours gives a good infiltration. Embedding is as usual.

Celloidin embedding may be accomplished in several ways. Dry tissues may be infiltrated in vacuo with absolute alcohol, and later transferred to thin celloidin dissolved in equal parts of alcohol and ether. This is gradually concentrated by the addition of dried celloidin. Dry tissues may be dropped directly into thin alcohol-ether celloidin, which after one day is concentrated. Or the dried tissues may be infiltrated with amyl acetate alone, or with a 3 per cent amyl-acetate solution of celloidin, in vacuo, or simply by diffusion. In any case, the celloidin is concentrated and the embedding ends with celloidin blocks containing tissue which may be cut in the usual way.

Mounting of the paraffin sections should be done by the dry method. The sections are flattened with the finger on slides previously covered with a thin film of albumen affixative. Each slide is warmed until the paraffin of the section is just melted and is then cooled as rapidly as possible. While the paraffin is just solidifying, the sections are again very carefully flattened.

E. Safety precautions

The procedure outlined above is not without danger to the operator and those about him. In all of this work it must be borne in mind that ammonia fumes in sufficient concentrations are toxic, that ammonia in lower concentrations is a heart stimulant, and that the susceptibility to ammonia is quite variable. In the course of the transfer of the ammonia to the Dewar flask surrounding the dehydrating chamber, the escape of ammonia fumes is unavoidable. Therefore, where possible the whole apparatus should be set up in a hood. If a hood is not available, it is necessary to have an electric fan to turn aside the ammonia fumes. Spattering of liquid ammonia during the transfer may cause burns if enough liquid ammonia comes in contact with the skin.

In addition to taking these precautions, it is necessary to bear in mind also the danger due to three possible sources of explosion. In the first place, liquid air forms explosive mixtures with some organic substances. This has never occurred in our experience with liquid air, but it is well to remember that it may occur. In the second place, liquid ammonia sometimes becomes superheated, and will suddenly boil with violent bumping, without warning. This occurred once in this laboratory when the glass stopper of the inlet tube was removed too soon after the pressure on the liquid ammonia was reduced. It may also occur after the transfer of liquid ammonia from the ammonia tank to the warmer Dewar flask used in a transfer of the liquid ammonia. Finally, an explosion may take place at any time with liquid air or liquid ammonia if Dewar flasks of soft glass are used. These explosions also occur spontaneously and without warning. We have had one explosion of this sort with liquid air in this laboratory.

As a precaution to be taken against these explosions, the use of Pyrex Dewars is advised whenever liquid air or liquid ammonia is used. Also, goggles should be worn during manipulations involving either fluid.

RESULTS

In the course of the development of the Altmann technique, we were engaged in the investigation of several aspects of fixation, histochemistry, and supravital staining. The chief purpose of these studies was to determine the limitations of the Altmann technique. The most convincing argument for or against the technique centers around whether or not it works when applied to specific problems. We have therefore been engaged on specific problems involving the application of the observations and conclusions to be elaborated in this section. The results of these investigations of specific problems will be published separately. They are to be regarded as evidence, supplementing that described in this section, of the value of the Altmann technique when applied to the fixation of tissues, histochemistry, and the permanent preparation of supravitally stained tissues.

A. Fixation

1. General considerations. All of the usual fixatives used in histology are in a fluid medium of some sort, and penetrate the organ by diffusion. Necessarily, at least three defects are notable. In the first place, if a fixative must penetrate the block by diffusion, and since diffusion of the fixative cannot be complete at once, it follows that there will be a difference between the fixation of the cells at the surface of the block and those toward the middle of the block, this difference being greater when the block is large. This difference in the appearance of cells at the periphery and the inside of a block is accounted for by, 1) postmortem changes in the cells reached too slowly by the fixative, and, 2) dilution and change in the composition of the diffusing fixative by the water in the organ and its dissolved salts.

A second defect inherent in fluid fixatives is the extraction of salts and lipoids and the change in composition of some of the salts and lipoids. Diffusion of alcohol into an organ, for example, is countered by diffusion of water from

the organ into the surrounding alcohol. Most of the salts of the body are soluble to a more or less appreciable extent in both fluids, and would tend to diffuse with the fluids out of the block to other parts. Lipoid staining is affected in some way by every fixative, even those which are not lipoid solvents. This indicates that their composition has been altered. Moreover, the aftertreatment of organs after fixation generally involves the use of some fat-solvent which extracts the lipoids more or less completely unless they have been changed.

Finally, a defect of fixation with fluid fixatives which seems to be peculiar to each fixative, is a certain degree of shrinkage of the organ as a whole, involving a distortion of the cells of the organ or of parts of the cell. To what extent the use of such fluids, as such, is responsibe for the changes of cells during and after fixation, and in the treatment preceding embedding, is unknown.

The evidence establishing the validity of these generalizations is well known to every histologist, and is part of the experience of every worker in the field. They are repeated here to emphasize the advantages of fixation by the Altmann technique. Fixation by the Altmann technique, as outlined in the previous section, does not involve the use of any reagents, is accomplished practically instantaneously throughout the whole block, avoids the extraction of salts or lipoids, and, in some cases, causes no shrinkage of the organ as a whole or of the cells of the organ. These generalizations would follow logically from the principles involved in the method.

That fixation by the Altmann technique actually does preserve very nearly the genuine relations of cells and parts of cells of the living organ (for some organs) will be shown later in this section. To appreciate this, it will be necessary to study first the nature of fixation by the Altmann method and some of the limitations of the method with respect to fixation.

The nature of the fixation by the Altmann technique was investigated by an experiment of this sort:

Several blocks of a kidney of a rabbit were placed in a test tube which was sealed carefully from the air and kept immersed for three days in liquid ammonia in a Dewar flask at —33°C. to —35°C. At the same time, several blocks of the kidney were frozen in liquid air and dehydrated at about —20°C. by the Altmann method. In addition, one block was placed in distilled water, and after four hours, fixed in absolute alcohol and embedded in paraffin, together with another block of kidney fixed in absolute alcohol directly after the death of the animal.

After this, one block each of the frozen material and of the frozen and dehydrated material was immersed in distilled water, in Tyrode solution, and in alcohol. After four hours of soaking, with occasional stirring, the blocks of kidney were all dehydrated in alcohol and embedded in paraffin. The filtrates in all cases were then tested for protein. Something that gave a positive reaction with Robert's reagent, acetic-acid test for albumen, xanthoproteic test, Biuret test, Millon's test, lead-nitrate test, and tannic-acid test was found in the filtrate of both the frozen material and the frozen and dehydrated material, in the distilled water and in the Tyrode solution. The evidence indicates, then, that not all of the proteins are made insoluble in water or Tyrode solution by the freezing alone or the freezing and dehydrating by the Altmann technique.

The sections confirm the chemical evidence and supply some additional information. Haematoxylin- and eosin-stained sections from the kidney tissues frozen, but not dehydrated, and then fixed in alcohol are not appreciably different from those fixed directly in alcohol after the death of the animal. But the kidney tissue frozen and dehydrated by the usual Altmann technique and then placed in alcohol reveals a superior type of fixation of the tubules; the brush border is retained in most of the proximal convoluted tubules. This indicates that the removal of water from the frozen tissues is more effective in fixation than freezing at low temperatures alone. It also appears that tissues may be fixed cytologically without a complete precipitation of the proteins of the tissue.

Another series of studies on these sections seems to us to be of some importance in histochemistry. The blocks of frozen tissue and the frozen and dehydrated tissue, after being placed in Tyrode solution or distilled water for four hours, and then dehydrated with alcohol and embedded, show in sections stained with haematoxylin and eosin typical degeneration pictures; that is, the nucleus is vacuolated or pyknotic, and the cytoplasm is homogeneous. Whole tubules are separated from their basement membrane and are shrunken and shriveled. These observations indicate that the so-called autolytic enzymes were not destroyed, and that the tissue proteins were not sufficiently changed by the freezing or the freezing and dehydration to resist the action of the enzymes when the tissue is soaked in water or Tyrode solution.

In general, we safely say that dehydration is more effective in fixation by the Altmann than freezing alone, that the proteins are not completely precipitated by this method of fixation, and that at least one type of enzyme is not destroyed by the procedure of the Altmann technique.

2. Peculiarities of fixation by the Altmann technique. All of the peculiarities of technique for the fixation of organs by the Altmann technique are empirical affairs, and must be ascertained by each investigator for his own problem. In other words, there is no known generalization that will aid in determining, 1) the time of dehydration of blocks of tissue of various volumes from one organ, let alone different organs; 2) how thick the blocks may safely be for complete dehydration and good fixation; 3) to what extent the capsule of an organ interferes with its dehydration; 4) whether all organs will be equally well fixed, or, if not, which will be superior in their fixation. These problems are intimately interrelated.

The time required to dehydrate blocks of various organs varies with the size of the blocks of tissue, the nature of the tissue, the number of blocks of tissue being dehydrated at once, the surface area of the blocks, and the nature of the capsule of the organ. There are probably numerous other variables which are not so obvious. As many as fifteen to

twenty blocks of tissue may be dehydrated at the same time if the slices are 1 to 2 mm. thick, or in the case the liver, 3 to 4 mm. thick. In general, where there is no dense capsule, the significant dimension seems to be the thickness. For example, a piece of rabbit's stomach 2 cm. long and 2 cm. wide may be dehydrated in thirty hours, whereas a piece of liver 1 cm. long, 0.5 cm. wide, and 0.5 cm. thick will require three or four days, and a piece of kidney of these dimensions will require even longer if one of the poles of the kidney is used. This difference in the time required for complete dehydration is probably related in some way to the surface area exposed. It becomes clear, then, that there is no way of determining the minimum time for dehydration, except by trial and error. In view of these variations in the time required for dehydration, there should be a large factor of safety in the time actually allowed for dehydration in vacuo, to avoid error from the minute, unknown complications that prolong the time required for dehydration.

In addition to the factors of size and surface, the nature of the organ being dehydrated also affects the time required for dehydration. For example, lobulated organs like the pancreas react like the stomach; that is, their virtual surface is very large. The liver is always very rapidly dehydrated. The intestines as a whole are quickly dehydrated. The kidney is dehydrated almost as rapidly as the intestines. The adrenal and the spinal cord require somewhat longer. Five days of dehydration at low pressures failed to dry completely a rabbit's adrenal whose capsule remained intact. Four days of dehydration failed to dry completely one pole of a rabbit's spleen whose capsule had not been injured except at the relatively small cut surface. In this case, dehydration took place largely from the cut surface, the capsular portion being barely dried; the inside of the block of the spleen was not at all dehydrated. The capsule seems to impede the vaporization of water from the block. In the discussion of the time required for dehydration, we have deliberately avoided making specific recommendations of any sort, because the factors

involved are so variable, and no quantitative relations are possible. The best guide is to determine the minimum time required for the dehydration of a block of tissue of the size or shape to be used in the investigation and then allow a wide margin beyond this time.

It is almost as difficult to recommend the optimum thickness of a block of tissue for the most successful fixation as it is to recommend a minimum dehydration time. The fixation of cells differs very appreciably at different depths of a single block, unless the block is thin. In those cases where the block is too thick, the cells become more and more shrunken and the cytoplasm and nucleus more vacuolated the farther removed from the surface they lie. The kidney tubules as a whole are found to be more and more shriveled, the liver cords are increasingly distorted, etc., as the middle of the block is approached. The rabbit's intestine, being thin, does not show this effect. The marginal part of the rabbit's ear is very well preserved. A lobulated organ like the pancreas reacts like a thin organ; that is, it reacts as though lobules of the pancreas were separated and dried. The only safe generalization regarding the thickness of a block of tissue that will give the most successful fixation is that the thinner the block, the more likely it is that fixation will be good.

Up to the present, we have not been equally successful with the fixation of all organs. In general, the liver, pancreas, and skin of the rabbit are rather well preserved; the kidney, jejunum, and duodenum are fairly well prepared; but nervous tissue is very poorly fixed. We are still trying to vary the technique so as to improve the fixation of tissues of high water content. Specific examples to illustrate the fixation of tissues by the Altmann technique will be described in the remainder of this section.

3. Examples of fixation by the Altmann technique. Rabbit's ear. The stratified squamous epithelium is very well fixed. The intercellular bridges are prominent. The eleidin granules are discrete. The hyaline cartilage of the ear is also nicely preserved—the capsules are prominent, the cells fill practi-

cally the entire space of the lacunae. The nucleus of the cartilage cell is round, occupies a relatively small volume of the cell, is dense, and appears as a finely stippled chromatin in a more uniform background of basic-staining nuclear sap. The dermis contains capillaries, arterioles, and venules which are beautifully preserved. They give an impression of fullness and completeness difficult to obtain from material fixed in other ways.

The fibers of the erector pili muscles in sections stained simply with haematoxylin and eosin show very clearly what look like myofibrillae with individual, condensed regions on each myofibril that may correspond to contraction waves.

Amblystoma liver. The parenchymatous cells are very well preserved. The cytoplasm is vacuolated, but the vacuoles are confined to one pole of the cell somewhat in the manner of a secreting cell of the pancreas. The vacuolization is very much more discrete than that of the same material fixed in formol-Zenker or in absolute alcohol. The opposite pole of the cell is neatly preserved and is homogeneous. The mitochondria are visible even in the unstained sections. The evidence showing that these are largely protein in nature will be presented in a later paper. There are also some basic-staining granules, rods, and filaments. The nuclei, in addition to the chromatin granules, have the rich, full appearance characteristic of nuclei well fixed by the Altmann technique.

Rabbit's pancreas. The lobules are beautifully preserved, with the sections in the unstained, clear condition looking very much like the fresh. The alpha and beta cells of the islands of Langerhans show their granules very plainly. The zymogen granules of the acinar cells are clearly preserved. In the unstained section the mitochondria are clearly visible. The stained sections give an impression of completeness that is rarely obtained from other well-fixed material.

B. Histochemistry

1. General considerations. The most difficult problems facing histochemistry are and have always been to identify the substances normally present in cells, or introduced into them, and to demonstrate their location in the cell during life. In order to be really convincing, a reliable histochemical test should conform to the following requirements:

a. The substance to be tested for must be prevented from moving away from the site occupied during life to any other part of the tissue or into the surrounding fixing medium. In other words, the substance must be made insoluble immediately to prevent its diffusion in the tissue or extraction from the tissue. For example, a common method of testing for uric acid involves the fixation of organs with absolute alcohol, in which uric acid is practically insoluble. The fallacy of the method lies in the fact that it is only after about two hours of fixation of small pieces of tissue in absolute alcohol that the tissue spaces really contain absolute alcohol. Until then, the water normally present in tissues still remains in the tissues, and since it dissolves uric acid and urates to some extent, permits the uric acid and urates to diffuse. This difficulty will be discussed at greater length at a later time. Suffice it to say that in experiments with uric acid, it is possible that uric acid may diffuse from its original site to neighboring parts of the tissue without being appreciably extracted by the alcohol.

In regard to the extraction of substances by the fixative used to make them insoluble, an example of error is seen in the quantitative data of Policard and Okkels. They demonstrated that 10 to 14 per cent of mineral matter is removed by the best methods available for precipitating it during the process of fixation.

No methods have been available up to the present that will prevent the diffusion and extraction of potassium, sodium, chlorine, ferrocyanide, or numerous other easily soluble substances.

In view of these considerations, doubt has been cast on the significance of the results of micro-incineration. The ash observed after incineration may and probably does represent a distribution of inorganic constituents which does not obtain during life. That the distribution has been altered will be demonstrated at another time.

b. The substance to be tested for, in addition to being precipitated before it moves from its original site, must not be destroyed or altered. In some cases, the substance tested for is so labile that either it is destroyed before it is reached by the fixative, in which it is insoluble, or else it is chemically changed by the fixative so that it loses its characteristic reactions. For example, although glycogen is almost completely insoluble in alcohol, there is no doubt at all that the usual methods of fixation of tissues for glycogen permit of some glycogenolysis.

The importance of the Millon test for tyrosin cannot be overemphasized, because it is the chief protein test at present available for histochemical work. We have been able to demonstrate that the Millon test on tissue fixed in the usual fixatives is far less intense than the Millon reaction obtained with the same material frozen and dried by the Altmann technique. Moreover, completion of the fixation of dehydrated material in alcohol reduces the strength of the Millon reaction. There is no doubt that in the course of fixation by the usual methods, the reactivity of the groups or configuration responsible for the formation of the pink color in the Millon test is affected. A similar reaction probably takes place with the substances responsible for Feulgen's nucleal reaction, for the color obtained is much more intense in dehydrated material than in the same tissue fixed in the usual way.

c. With the substance to be tested for precipitated in a reactive state in the position occupied by it during life, it is necessary to make the substance visible in some way, and still prevent it from diffusing out during the application of the reagent used for this purpose. This would mean that

an exceedingly insoluble and colored precipitate of some sort is necessary. The color reaction must be specific for the substance tested for. A further requirement of an adequate histochemical technique is that the colored substance should be in such a state that it will not tend to move from the place originally deposited to be adsorbed by other surfaces. These considerations are very important, but are not related directly with the Altmann technique. They will therefore not be elaborated in this paper.

The Altmann technique effectively satisfies the requirements of fixation of tissues for histochemical studies by causing, 1) the precipitation of all of the solid constituents of tissues without their extraction or diffusion, and, 2) the retention of these substances in a reactive state and chemically unchanged.

By the Altmann technique there is no known extraction of any solid constituent of tissues except lipoids, which are affected only by the embedding. At present, the only medium for embedding for histochemical purposes is paraffin. During the three to five minutes required for infiltrating in paraffin directly, it is practically certain that some lipoids are extracted while the paraffin is melted. Aside from the lipoids, no other constituent is appreciably soluble in paraffin, as far as we can detect. The extraction or diffusion of substances other than lipoids during the infiltration and embedding in paraffin may be considered absent or negligible. The only other time that the tissues are in a liquid phase (when diffusion may take place) is in the interval between the time the animal is killed and the tissue is removed and frozen in liquid air. There is some evidence that some diffusion occurs even during this brief interval. It is obvious that only the first one or two slices of an organ rapidly removed from an animal would yield the most reliable results, at least when rather soluble substances are being investigated.

The salts and liqoids of the tissue are not at all changed after dehydration by the Altmann technique, but the proteins are somewhat altered. The proteins, however, are probably

far less changed after fixation by the Altmann method than after the fixation, hardening, and dehydration of the usual methods. This is evidenced by the much more intense Millon reaction obtained with Altmann-fixed tissue, as mentioned above, and by the fact that not all proteins are rendered insoluble in water as a result of the Altmann technique. The proteins are probably altered during the embedding by the relatively high temperature to which they are subjected, so that, after sectioning, the proteins have been altered by the freezing, the dehydration, and probably by the heat of the paraffin.

The tissue, though fixed, is without a doubt, far closer to the living cell as regards the disposition and state of the solid constituents than can be obtained by any other method developed up to the present period.

The scientific basis for the method and the logical assumptions as to the state of the tissue substances are not as convincing as the actual experimental comparative results, some of which will be given here.

2. Applications of the Altmann technique to histochemistry. Uric acid. Uric acid is injected intravenously into a rabbit, and after some time the kidney is removed. Very thin slices of the kidney are cut. Some are fixed rapidly in alcohol; others are frozen and dehydrated by the Altmann technique. After sectioning and staining for uric acid, there is a tremendous difference in the appearance, distribution, and quantity of the uric acid in the sections from the blocks fixed in alcohol, and those fixed by freezing and drying. The crystals in the alcohol-fixed sections are large, nucleated, and formed; they are very numerous in the proximal convoluted tubules, the loop of Henle, the distal convoluted tubules, and the collecting ducts. The frozen and dried sections show only rarely, chiefly in the collecting ducts, the type of crystal described above, most of the uric acid existing as a finely dispersed powder in the lumen of the tubules and ducts, and in Bowman's space. The crystals of uric acid are absent from the glomerular space of alcohol-fixed sections.

Moreover, the brown-black deposit of reduced silver (representing uric acid) is more closely confined in the dried material; the uric acid has stained the nuclei, cytoplasm, and connective tissue of neighboring tubules in the alcohol-fixed material. On the whole, the quantitative and morphological aspects of uric acid in the kidney are plainly more accurate and closer to the state existing during life than that shown by the standard method involving the use of alcohol.

Ferrocyanide. Similar comparative results were obtained with the kidney of animals injected with a ferrocyanide when some blocks of the kidney were fixed by the Altmann method and other blocks after perfusion of the animal through the aorta with a copper-sulphate solution.

Glycogen. Some glycogenolysis is considered to take place even with optimal fixation of the tissue for glycogen. Alcohol is generally used as a fixative, glycogen being insoluble in it. But the distortion caused by alcohol is so great that the usual picture of glycogen in the liver, for example, is that of a crescent or of granules confined to one pole of the cell. So that presumably, during fixation, there is some movement of the glycogen which has remained after escaping glycogenolysis. At present, tissues are analyzed quantitatively for glycogen by freezing the organ in liquid air, grinding while frozen, and mixing with chilled alcohol. This is designed to prevent glycogenolysis by inhibiting it during the freezing, and destroying the 'glycolytic enzymes' with alcohol while they are still inactive. Essentially, the same procedure is followed during fixation by the Altmann technique, the glycolytic enzyme being inhibited by freezing and drying. Sections of liver run through by the Altmann technique show a diffuse glycogen reaction, indicating that the glycogen is distributed generally through the cell.

C. Permanent preparation of supravitally stained organs

All that has been said about fixation and histochemistry applies equally well to the preparation of supravitally stained organs. The ideal permanent preparation of an organ

supravitally stained would be one which has retained the distribution of the supravital dye that it had during life in the well-fixed organ. This means that the precautions necessary for the most efficient fixation outlined in the previous section must be rigorously observed.

There are three possible periods of diffusion of the dye. The first is relatively short, and is the period between the killing of the organ and the freezing of the slice of tissue in liquid air. The second is the peroid of infiltration, when the paraffin medium is melted; this period may occupy three to five minutes. Both of these periods of possible movement of the dye are discussed in the previous section on histochemistry.

The last possible source of error is involved in the removal of the paraffin from the sections and the mounting of the sections. For accurate work both the deparaffinating agent and the mounting medium must be liquids which will not dissolve or permit movement of the dye being investigated.

In cases where it is difficult to find a satisfactory mounting medium and it is necessary to mordant the dye, infiltrating the block of tissue in vacuo after dehydration with a solution of the mordant is the most efficient procedure.

CONCLUSIONS

1. Some forty years ago Altmann, in connection with his cytological work on bioblasts, stated that biological material could be dried without shrinkage at a temperature of —20°C. The statement of Altmann in this respect has been confirmed and an apparatus and procedure has been devised for carrying out the process.

2. Fixation of some organs and tissues (skin, cartilage, smooth muscle, liver, pancreas) is excellent cytologically; fixation of other organs is less satisfactory; central nervous system is exceedingly poorly fixed.

3. The Altmann technique has been successfully applied to the investigation of the excretion of uric acid and ferrycyanide, the chemical nature of intracellular constituents, and of the intracellular distribution of glycogen.

4. The method has also been used for the preservation of vitally or supravitally stained preparations which fade in fixation or are incapable of satisfactory preservation by other means.

LITERATURE CITED

ALTMANN, R. 1890 Die Elementarorganismen und ihre Beziehungen zur den Zellen. Leipzig: Veit.

BAYLISS, W. M. 1924 Principles of general physiology. New York: Longmans, Green & Co.

DUNOYER, L. 1926 Vacuum practice. London: G. Bell & Sons, Ltd. Translated by J. H. Smith.

DUSHMAN, S. 1922 The production and measurement of high vacuum. Schenectady, New York: General Electric Review.

HOAG, J. B. 1929 Electron physics. New York: D. van Nostrand Company. Appendix B, C, D.

MANN, G. 1902 Physiological histology. Oxford: Clarendon Press.

MATHEWS, A. P. 1925 Physiological chemistry. 4th ed. New York: W. Wood & Co.

NEWMAN, F. H. 1925 The production and measurement of low pressures. New York: D. van Nostrand Company.

POLICARD, A., AND H. OKKELS 1930 Localizing inorganic substances in microscopic sections. The microincineration method. Anat. Rec., vol. 40.

ROMEIS, B. 1928 Taschenbuch der mikroskopischen Technik. München und Berlin: Oldenbourg.